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13. ABSTRACT (Maximum 200 words) This project is aimed at developing specific and effective vaccines for immunotherapy of breast cancer. Mutated epidermal growth factor receptor (mEGF-R) is expressed on a high proportion of breast carcinoma lesions derived from various patients, but not on any normal tissues tested, and is targeted in these studies. The major goal is to develop a rat model of immunotherapy against mammary carcinoma targeting mEGF-R. In this model vaccines of mEGF-R (peptides, anti-idiotypic antibodies, recombinant antigen) are tested for their effects on established tumor growth. Towards these goals, rat mammary carcinoma cells were transfected with rat mEGF-R cDNA and characterized. The transfectants express mEGF-R protein and MHC class I and II antigens and are tumorigenic in syngeneic rats. To develop anti-idiotypic antibody and recombinant antigen vaccines of rat mEGF-R, recombinant rat mEGF-R protein was produced in baculovirus and characterized. Five peptides mimicking rat mEGF-R also were produced for vaccine purposes. Thus, the goals originally proposed for the first year of study have been fulfilled and the rat model can now be used to evaluate the potential of mEGF-R vaccines to induce humoral, cellular and protective immunity.				
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FOREWORD

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TARGETING MUTATED EPIDERMAL GROWTH FACTOR RECEPTOR

Table of Contents

Foreword..... 3

Introduction 5

Body of Work 5

Conclusions..... 7

References..... 7

Appendices..... 10

Introduction

Clinical trials of active immunotherapy in breast carcinoma patients have suffered from the use of vaccines that induce only humoral, but not cellular, immunity (1,2) or lack specificity (3). Preclinical and clinical studies with cancer vaccines have demonstrated a correlation between the induction of humoral or cellular immunity and tumor growth inhibition (4-12). Thus, tumor vaccines ideally should induce both humoral and cellular immunity and induced immunity should be specific for the tumor cells. The major goal of this study is to test tumor-specific vaccines against breast carcinoma in a relevant rat model. Mutated epidermal growth factor receptor (mEGF-R) is expressed by a high proportion of breast carcinoma tissues derived from various patients, but not several normal tissues tested (13; and our unpublished data described in the original proposal). mEGF-R is expressed both on the surface and in the cytoplasm of tumor cells (13), rendering it a target for both B and T cells. Furthermore, targeting of mEGF-R may exert direct anti-proliferative effects (14).

We have chosen a rat model of mEGF-R vaccines for the proposed studies because of the availability of cloned normal rat EGF-R (15) and MHC class I and II positive rat mammary carcinoma cells with either high or low metastatic capability (16,17). The most specific vaccine of mEGF-R consists of the minimal sequence, including the mutation, that elicits B- and/or T-cell responses. We have chosen peptides of mEGF-R for induction of T cells, analogous to studies performed successfully with peptide vaccines by other groups (18-24) and our collaborators (25-27) in various antigen systems. Anti-idiotypic antibody vaccines will be produced to induce B cell immunity to mEGF-R. Our studies have demonstrated that anti-idiotypes can induce humoral, cellular and protective immunity in colorectal cancer patients (28-30). Molecular cloning of anti-idiotypic antibodies recently developed in our laboratory (31,32) has numerous advantages over traditional approaches, such as high sensitivity, specificity and ease and economy of production.

In conclusion, mEGF-R is a unique target for active specific immunotherapy of breast carcinoma, based on its specificity, frequency of expression, potential for activating both B and T cells, and availability of an ideal animal model of active immunotherapy. The studies will provide the rationale for specific active immunotherapy of breast carcinoma patients. The results we will obtain with mEGF-R in the rat mammary carcinoma model may be applicable to other tumor systems, such as lung carcinomas and gliomas which also express mEGF-R (13,33).

Body of Work

During the first year of funding (July 96-June 97), our efforts focused on the generation of rat mammary carcinoma cells expressing rat mEGF-R. These cells are needed for the proposed studies on the induction of humoral, cellular and protective immunity against breast carcinomas targeting rat mEGF-R. For transfection of the cells with rat mEGF-R cDNA, we used an expression vector that had been produced before in our laboratory and has been described in detail in the original application. Rat mammary carcinoma cells MTLN3 were transfected with PcDNA3-rat-mEGF-R vector containing G418 selectable marker. Stable transfectants were selected with G418 and cloned. Eight clones were frozen and one of them (MTLN3-pc/RmEGF-R) was characterized in detail. Both the parental MTLN3 cells and the transfectants reacted in FACS analysis of intact cells with rabbit serum specific for the extracellular domain of normal rat EGF-R; transfectants demonstrated higher serum reactivity than the parental cells (Fig. 1 in Appendix). However, monoclonal antibody L8 that specifically binds to human mEGF-R did not react with the transfectants, although it did react with murine cells expressing transfected human mEGF-R (results not shown). Therefore, we investigated by Western blot analyses whether the transfectants express the mutated protein. The 145 kDa band characteristic of mEGF-R was indeed detected in the transfectants using rabbit antibody to normal rat mEGF-R (intracellular domain) (Fig. 2, Appendix), whereas no such band was

detected when MAb L8 was used (not shown). As expected, the 170 kDa band characteristic of normal rat EGF-R was also detected. Western blot analysis of membrane extracts did not reveal presence of the 145 kDa rat mEGF-R protein (data not shown). Thus, the inability of monoclonal antibody L8 to react with the transfectants by FACS and Western blot analysis may be due to the apparent differences in the amino acid sequences between the rat and human mutated epitopes. Alternatively, the mutated epitope may not be correctly expressed by the transfectants. We are currently sequencing the transfected mEGF-R cDNA isolated from the cells by RT-PCR. The transfectants were further characterized for their tumorigenicity in syngeneic rats following injection of the cells into the mammary fat pad. The transfectants were significantly ($P < 0.05$; Student's t-test) more tumorigenic than the parental cells using identical numbers of cells in the inoculum (Fig. 3, Appendix), confirming a previous report (14).

A major goal of our initial studies was to produce vaccines of rat mEGF-R for use in later experimental active immunotherapy studies. These vaccines comprise anti-idiotypic antibodies and peptides, both mimicking the mEGF-R epitope. Monoclonal antibody reactive to the rat mEGF-R epitope is needed as the basis for production of anti-idiotypes. Since monoclonal antibody L8 to the human mEGF-R epitope did not react with the rat mammary carcinoma cells transfected with rat mEGF-R cDNA, although they expressed the mEGF-R protein (see above), we decided to produce monoclonal antibody to rat mEGF-R protein in mice. As a first step toward this goal, we produced rat mEGF-R protein in baculovirus for immunization of mice in order to obtain a monoclonal antibody against the mutated epitope. A baculovirus expression vector for rat mEGF-R was constructed and the presence of the correct insert was confirmed by sequencing (Fig. 4, Appendix). Recombinant baculovirus was produced and used to infect insect cells. The supernatant of the infected insect cells was tested by Western blot analysis for the presence of the mutated protein (extracellular domain) at 24, 48 and 72 hr after infection of the cells, using monoclonal antibody NCL-EGF-R to the extracellular domain of normal human EGF-R as a probe. The results are presented in Figs. 5 and 6 (see Appendix). Monoclonal antibody NCL-EGF-R reacted with the 41 kDa band characteristic of the extracellular domain of rat mEGF-R in 24 hr supernatants (Fig. 5, lane 6); additional bands were seen in the 48 hr and 72 hr supernatants, probably reflecting reactivity of the antibody with enzymatically degraded protein (Fig. 5, lanes 4 and 5). Therefore, rat mEGF-R protein was purified from 24 hr supernatants and tested in Western blot using NCL-EGF-R antibody. The 41 kDa rat mEGF-R protein was predominantly detected (Fig. 5, lane 2). The protein was then tested in ELISA for reactivity with monoclonal antibody NCL-EGF-R to normal human EGF-R and monoclonal antibody L8 to mutated human EGF-R. As expected, the purified rat protein reacted specifically with monoclonal antibody NCL-EGF-R, but not with control mouse myeloma protein (Fig. 6, Appendix). NCL-EGF-R did not react with BSA control protein, confirming the specificity of the reaction. To our surprise, rat mEGF-R protein also specifically reacted with monoclonal antibody L8 in spite of the absence of reactivity of this antibody with mEGF-R transfected rat mammary carcinoma cells (see above).

These results suggest that the mutated epitope may be intact when presented by the recombinant protein, but may not be expressed correctly in the cells transfected with mEGF-R cDNA, although these cells express a protein of the size characteristic of mEGF-R. This emphasizes the need for sequencing the mutated protein in the transfectants (see above). If the transfectant we have characterized thus far does not express rat mEGF-R epitope, we will test the other 8 transfected clones for reactivity with monoclonal antibody L8. If positive, clones will be characterized in detail by Western blot analysis and mEGF-R will be sequenced.

Five peptides mimicking rat mEGF-R were selected for vaccine purposes and are described in detail in Table 1.

Human mutated EGF-R was also produced in baculovirus by constructing a vector similar to the one constructed for expression of rat mEGF-R (see Fig. 4). The human mutated

EGF-R protein is needed for the evaluation of breast carcinoma patients' lymphocyte reactivities with the mutated protein (Specific Aim 3 of the original proposal). The characteristics of the mutated human protein are shown in Figs. 5 and 6. Western blot analyses of purified human mEGF-R protein derived from baculovirus revealed the 30 kDa protein characteristic of the extracellular domain of human mEGF-R (Fig. 5, lane 1). Analysis of the purified protein in ELISA shows its reactivity with both monoclonal antibody NCL-EGF-R to human normal EGF-R and antibody L8 to human mEGF-R (Fig. 6). The specificity of this reaction is emphasized by the absence of reactivity of control mouse myeloma protein with human mEGF-R protein and monoclonal antibody NCL-EGF-R with control BSA (Fig. 6).

Conclusions

During the initial funding period, our efforts focused on the establishment of the rat model of active specific immunotherapy against mEGF-R, a breast carcinoma-specific antigen. Rat mammary carcinoma cells expressing rat mEGF-R and vaccines of the mutated protein needed to be developed in order to evaluate in subsequent studies the vaccine effects against growth of mEGF-R positive mammary carcinoma cells in rats. Rat mammary carcinoma cells were transfected with rat mEGF-R cDNA and a transfected clone was characterized. The transfectants expressed the mutated protein, in addition to the constitutively expressed normal EGF-R protein, as determined by Western blot analyses of whole cell extracts. The transfectants were tumorigenic in syngeneic rats. Sequencing of the mutated protein will confirm expression of the mutated epitope. To develop mEGF-R vaccines, peptides and recombinant proteins (for production of anti-idiotypic vaccines) were produced in baculovirus and expression of the mutated epitope by the protein was confirmed. Thus, we have fulfilled the goals originally proposed for the first year of study. The model we have established will be useful for the evaluation of mEGF-R tumor-specific vaccines against mammary carcinoma in our subsequent studies.

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FACS analysis of MTLN3 rat mammary carcinoma transfectants for rat EGF-R expression

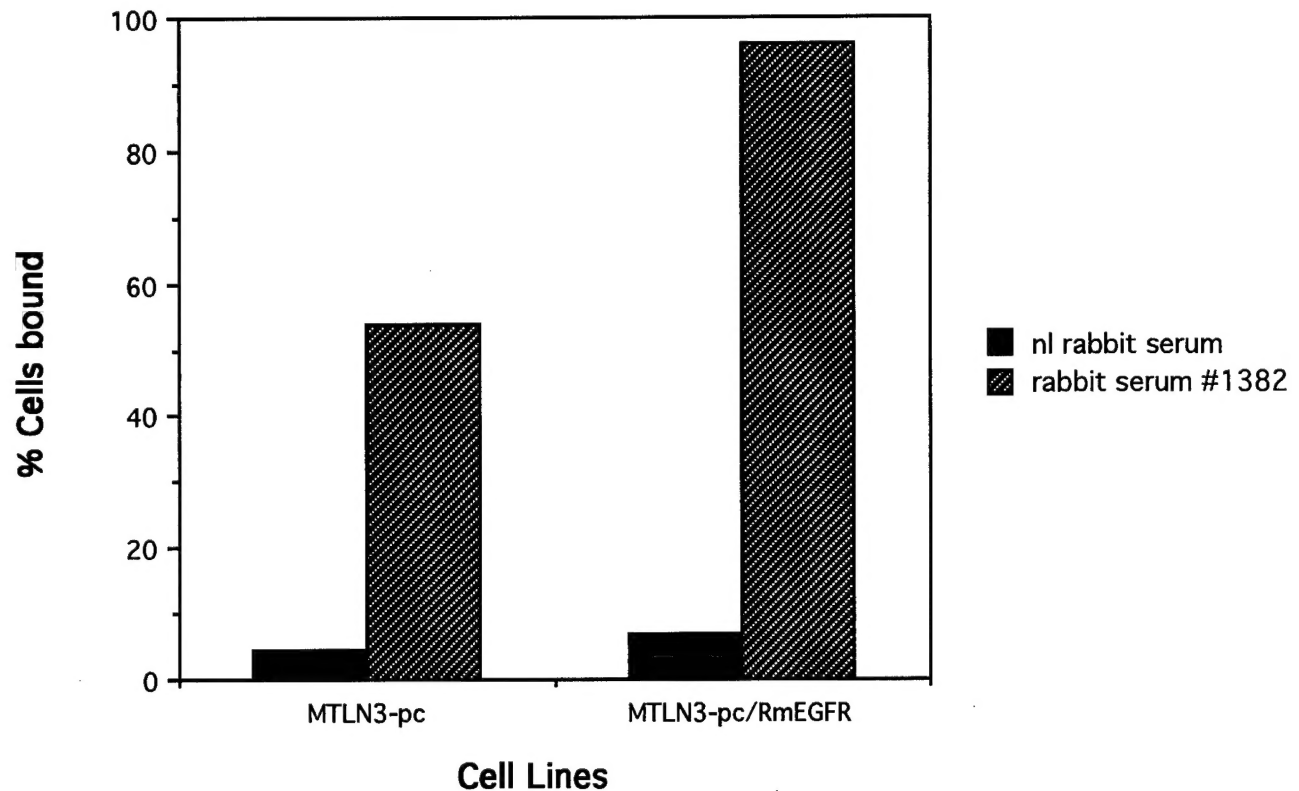


Fig.1. FACS analysis of rat EGF-R expression in MTLN3 transfectants. MTLN3-pc or MTLN3-pc/RmEGF-R cell lines were incubated with normal (nl) rabbit serum or rabbit serum #1382 (1:50 dilution) against normal rat EGF-R extracellular domain. FITC-labeled goat anti-rabbit IgG (1:200 dilution) was used for FACS analysis. Serum #1382 binds significantly higher to MTLN3-pc/RmEGF-R cells expressing both nl and mutated EGF-R compared to MTLN3-pc with only nl EGF-R.

Expression of rat mutated EGF-R in transfected rat mammary carcinoma cells MTLN3

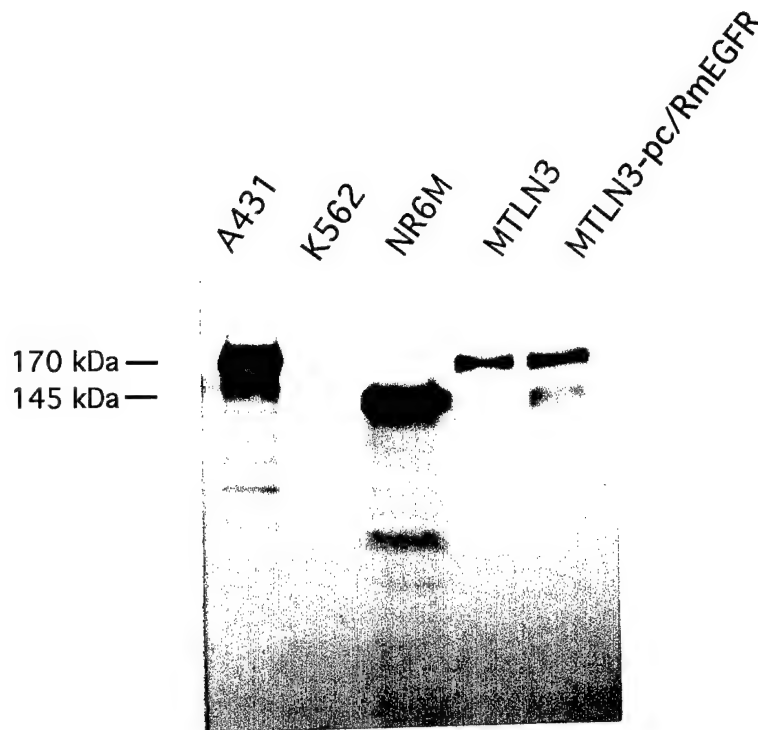


Fig.2. Western blot analysis of rat (R) mutated (m)EGF-R expression by transfected MTLN3 rat mammary carcinoma cells. Lysates (100 ug of protein) of cell lines MTLN3-pc/RmEGF-R; MTLN3-pc; negative control cells K562, and 10 ug of lysate of positive control epidermoid carcinoma cells A431 and NR6M cells transfected with human (m)EGF-R were separated on 7.5% SDS-PAGE gel and transferred to nitrocellulose. The membrane was blocked with 5% dry milk in PBS with 0.05% of Tween 20 and incubated with rabbit serum #22 (diluted 1:1000) against rat EGF-R (specific for intracellular domain) and receptor expression was detected by chemoluminescent reaction with HRPO-labeled goat anti-rabbit IgG diluted 1:3000. A 170 kDa normal EGF-R protein was detected in all lines except K562 cells, and the 145 kDa mutated EGF-R protein was detected in the MTLN3-pc/RmEGFR-R and NR6M transfectants, but not in parental MTLN3 cells.

Tumorigenicity of MTLN3 and MTLN3-RmEGF-R transfectants

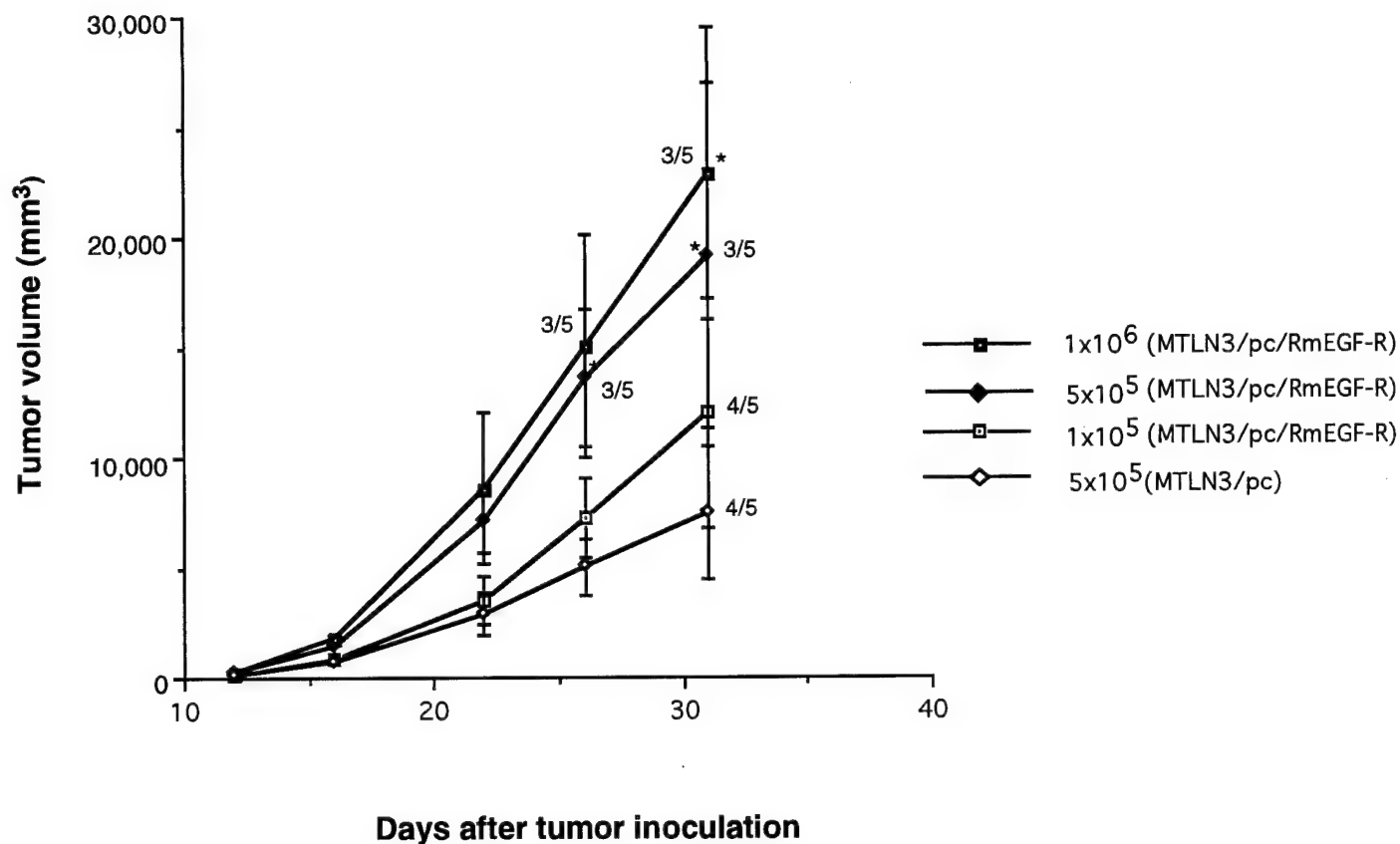


Fig.3. Tumor growth following mammary fat pad inoculation of MTLN3-pc and MTLN3-pc/RmEGF-R cells. Fisher 344 rats (5/group) were inoculated with either MTLN3-pc (5×10^5 cells/rat) or MTLN3-pc/RmEGF-R cells (1×10^5 to 1×10^6 cells/rat) at three different doses. Tumor volumes were recorded at weekly intervals. Stars represent significant differences ($P < 0.05$) between MTLN3-pc group and either the 1×10^6 or 5×10^5 MTLN3-pc/RmEGF-R.

Recombinant baculovirus rat mutated EGF-R expression vector

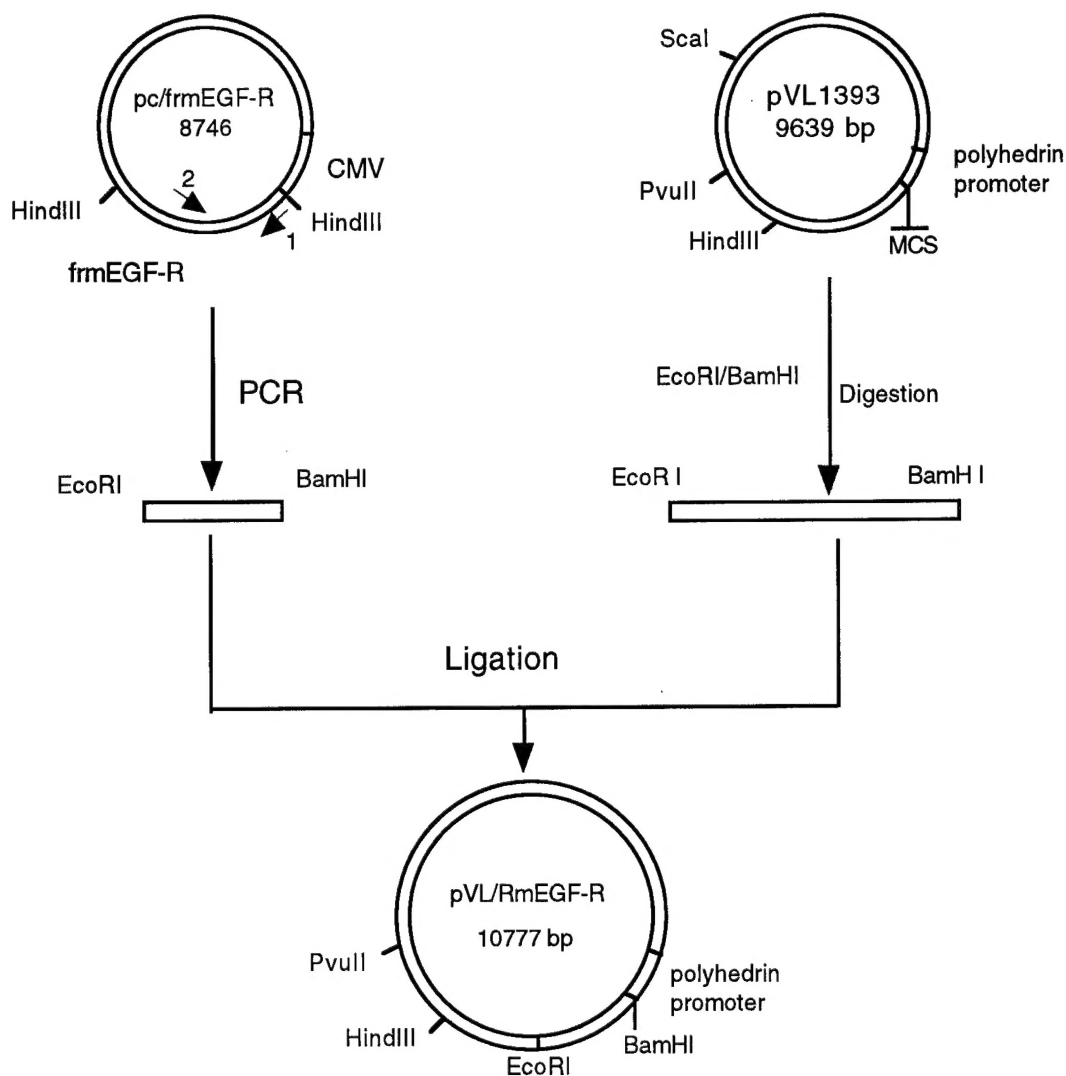


Fig.4. Construction of recombinant baculovirus rat mutated EGF-R expression vector. A 1.1 kb PCR product with extracellular domain from plasmid DNA pc/frmEGF-R was ligated into EcoRI/BamHI site of pVL1393 baculovirus vector. The insert was confirmed by sequencing and restriction enzyme digestion. MCS: multiple cloning site.

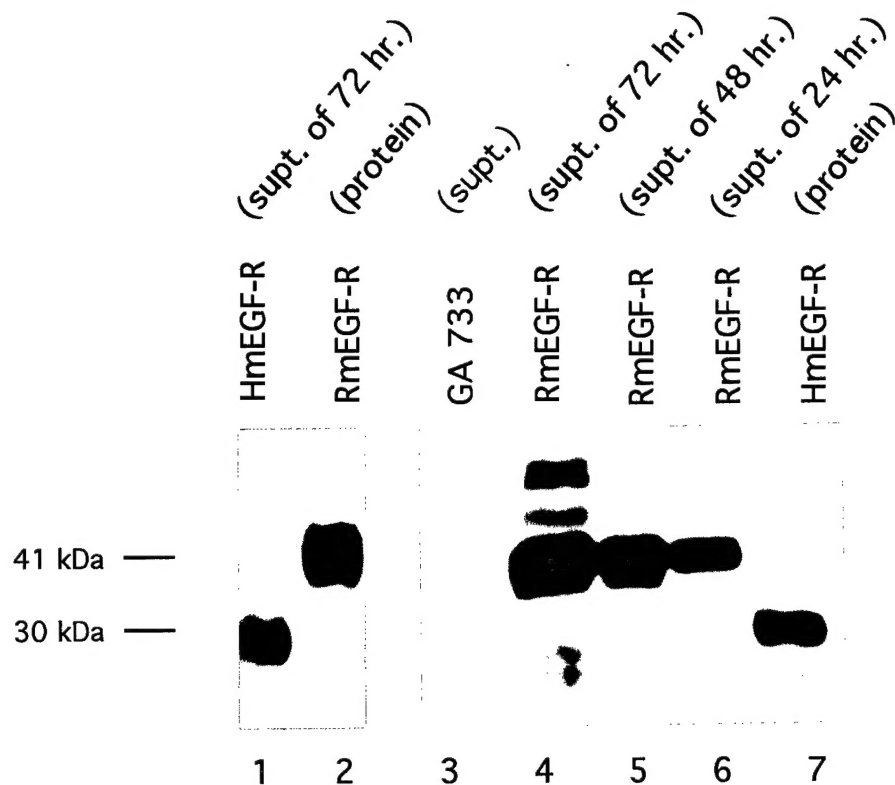


Fig. 5. Western blot analysis of recombinant extracellular domain of human (H) mutated (m) EGF-R (272 aa. residues 1-539) and rat (R) mEGF-R (379 aa. residues 1-646) proteins derived from recombinant baculoviruses. 10 μ l of supernatant from insect cells infected with recombinant HmEGF-R baculovirus (lane 1), 0.1 μ g of purified RmEGF-R protein (lane 2), 10 μ l of negative control GA 733 supernatant (lane 3), 10 μ l of supernatant from insect cells infected with recombinant rat mutated EGF-R baculovirus and collected at 72 hr (lane 4), 48 hr (lane 5), and 24 hr of incubation (lane 6), and 0.1 μ g of the affinity purified human mutated EGF-R protein (lane 7) were separated on 12% SDS-PAGE gel, and transferred to nitrocellulose. The membrane was subsequently incubated with mAb to human EGF-R extracellular domain (NCL-EGFR, 1:50 dilution), and antibody binding was demonstrated by chemoluminescence reaction of HRPO-labeled goat anti-mouse IgG (1:2,000 dilution). The approximately 30 kDa HmEGF-R protein (lane 1,7) and 41 kDa RmEGF-R protein (lanes 2,4,5,6) are shown. No band was detected in GA 733 control supernatant (lane 3).

Baculovirus-derived rat mEGF-R protein

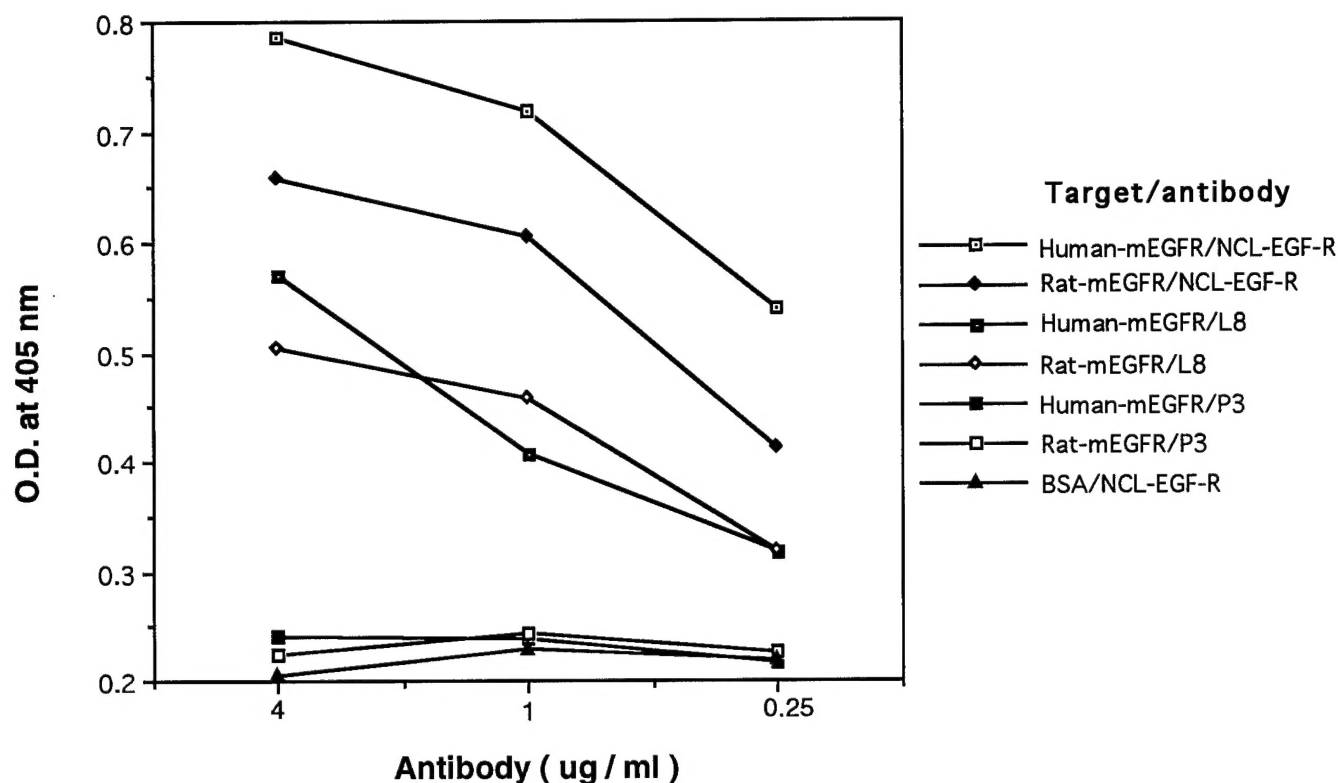


Fig.6. Characteristics of rat mEGF-R protein in ELISA. Purified rat mEGF-R, positive control human mutated EGF-R (2 ug/ml) derived from recombinant baculovirus or BSA control protein (0.1%) were coated onto microtiter wells. mAb NCL-EGFR (targeting normal human EGFR extracellular domain), L8A4 targeting mutated human EGF-R or P3 mouse myeloma protein (negative control) were added, followed by incubation with POD-goat anti-mouse IgG (1:5000 dilution). Both the human and the rat mEGF-R proteins significantly bound to both mAb NCL-EGFR and mAb L8A4.

Table 1. Peptides of Rat mEGF-R

Residues	Peptide		Hypothetical peptide patterns ^a		
	Amino acid sequence ^b	MHC class I binding motif (C-terminal) ^c	Rothbard pattern	α -amphipathic helix	
24-32	AL <u>EE</u> KK <u>G</u> NY	32	23-26	-	
25-33	LE <u>EE</u> KKGNVY	33	-	-	
27-35	EKKGNVYVVT	35	-	-	
22-38	GGA <u>LEE</u> KKGNVYVTDHG	Multiple	23-26	-	
12-38	LLLLAALLAPGGAL <u>EE</u> → KKGNVYVTDHG ^d	Multiple	23-26	-	

^a Based on human and murine peptide sequence analysis (35); numbers refer to residues.

^b Mutated glycine is underlined. Control peptides are selected for each rat mEGF-R peptide that induces a mEGF-R-specific immune response by omitting glycine (position 30) from the specific peptide or by replacing glycine by either proline or glutamic acid.

^c C-terminal MHC class I anchors of peptides are often hydrophobic or charged. For peptides > 9 amino acids in length, C-terminal anchors may vary depending on peptide cleavage in the proteasome. Additional anchors are usually highly variable in mice and humans and no predictions can be made for rats.

^d Leucine and arginine residues are often involved in MHC class II binding in humans (35).